表１　CTX-M 遺伝子検出、型別、シークエンス用プライマー

名称	配列（5'→3'）	増幅断片
CTX-MU1	ATG TGC AGY ACC AGT AAR GT	
CTX-MU2	TGG GTR AAR TAR GTS ACC AGA	593bp
CTX-M1GF	ATG GTT AAA AAA TCA CTG CG	
CTX-M1GR	TTA CAA ACC GTA GGT GAC	876bp
CTX-M2GF	ATG ATG ACT CAG AGC ATT CGC C	
CTX-M2GR	TCA GAA ACC GTG GGT TAC	876bp
CTX-M9GF	ATG GTG ACA AAG AGA GTG CAA	
CTX-M9GR	TCA CAG CCC TTC GGC GAT	876bp
CTX-M1GFJYM13Fmod	TT AC TGT AAA ACG ACG GCC AGT ATG GTT AAA AAA TCA CTG CGY C	
CTX-M1GRJYM13Rmod	TT AC CAG GAA ACA GCT ATG ACC TTA CAA ACC GTC GGT GAC GAT	920bp
M13Fmod60	AC TGT AAA ACG ACG GCC AGT	—
M13Rmod60	AC CAG GAA ACA GCT ATG ACC	—

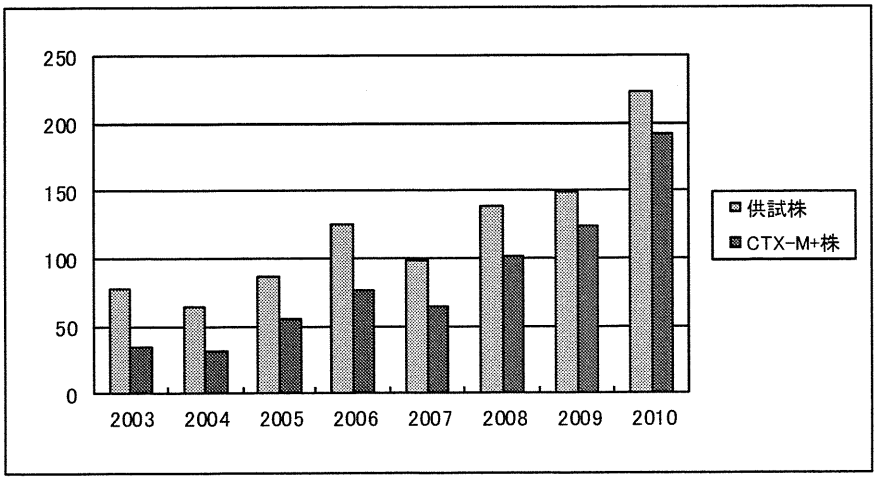

図１　秋田県における CTX-M 遺伝子陽性株の検出状況（2003.1.24-2010.12.31）

表２　CTX-M 遺伝子陽性株（680 株）の由来

由来	株数	由来	株数
不明	379	膣	3
尿	206	腹水	3
喀痰	41	胆汁	2
血液	19	咽頭	1
膿	11	子宮	1
便	4	耳漏	1
創部	4	髄液	1
浸出液	3	泌尿器	1

表３　CTX-M 遺伝子陽性株（680 株）の菌種

菌種	株数	菌種	株数
菌種不明	178	*K. oxytoca*	1
E. coli	257	*E. aerogenes*	1
P. mirabilis	212	*Providencia* spp.	1
K. pneumonia	22	*Klebsiella* spp.	1
P. stuartii	4	*C. broakii*	1
P. vulgaris	1	*M. morganii*	1

表４　CTX-M 遺伝子の型別結果

	CTX-M型					
菌種	1	2	9	2/9	UT	計
E. coli	36	12	49	4	2	103
P. mirabilis		45			1	46
K. pneumoniae		3				3
C. broakii		1				1
M. morganii		1				1
菌種不明		6	20		1	27
計	36	68	69	4	4	181

Ⅲ.　研究成果の刊行に関する一覧表

平成 23 年　研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
N. Nagano, Y. Nagano, M. Toyama, K. Kimura, T. Tamura, K. Shibayama, Y. Arakawa	Nosocomial spread of multidrug-resistant group B streptococci with reduced penicillin susceptibility belonging to clonal complex 1	The Journal of Antimicrobial Chemotherapy	In press		2012
K. Kimura, N. Nagano, Y. Nagano, J. Wachino, S. Suzuki, K. Shibayama, Y. Arakawa	Predominance of sequence type 1 group with serotype VI among group B streptococci with reduced penicillin susceptibility identified in Japan	The Journal of Antimicrobial Chemotherapy	66	2460-4	2011
Wachino J, Yoshida H, Yamane K, Suzuki S, Matsui M, Yamagishi T, Tsutsui A, Konda T, Shibayama K, Arakawa Y.	SMB-1, a novel subclass B3 metallo-β-lactamase, associated with ISCR1 and a class 1 integron, from a carbapenem-resistant *Serratia marcescens* clinical isolate.	Antimicrob Agents Chemother	55	5143-9	2011
Wachino J, Yamane K, Arakawa Y.	Letters to the Editor Practical disk-based method for detection of *Escherichia coli* clinical isolates producing the fluoroquinolon e-modifying enzyme AAC(6')-Ib-cr.	J Clin Microbiol.	49	2378-9	2011
A. Tsutsui , S. Suzuki , K. Yamane , M. Matsui , T. Konda , E. Marui , K. Takahashi , Y. Arakawaa	Genotypes and infection sites in an outbreak of multidrug-resistant Pseudomonas aeruginosa	Journal of Hospital Infection	78	317-322	2011
H. Yamashita, H. Tomita, T. Inoue and Y. Ike	Genetic Organization and Mode of Action of a Novel Bacteriocin, Bacteriocin 51: Determinant of VanA-Type Vancomycin-Resistant *Enterococcus faecium*.	Antimicrob. Agents Chemother.	55(9)	4352-4360	2011
H. Tomita, Jang-Jih Lu and Y. Ike	High Incidence of Multiple-Drug Resistant Pheromone-Responsive Plasmids and *Evidence of Transmissions* of VanA-Type Vancomycin-Resistant Enterococcus faecalis from Livestock to Human.	Antimicrob. Agents Chemother.			Revised

Suzuki M, Yamada K, Nagao M, Aoki E, Matsumoto M, Hirayama T, Yamamoto H, Hiramatsu R, Ichiyama S, Iinuma Y	Antimicrobial ointments and methicillin-resistant *Staphylococcus aureus* USA300.	Emerg. Infect. Dis.	17	1917-1920	2011
Kitao T, Miyoshi-Akiyama T, Tanaka M, Narahara K, Shimojima M, Kirikae T.	Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo-β-lactamases that mediate carbapenem resistance in *Pseudomonas.*	J Microbiol Methods.	87(3)	330-337	2011
Tada T, Kitao T, Miyoshi-Akiyama T, Tanaka M, Kirikae T.	Genome sequence of multidrug-resistant *Pseudomonas aeruginosa* NCGM1179.	J Bacteriol.	193(22)	6397	2011
Miyoshi-Akiyama T, Kuwahara T, Tada T, Kitao T, Kirikae T.	Complete genome sequence of highly multidrug-resistant *Pseudomonas aeruginosa* NCGM2.S1, a representative strain of a cluster endemic to Japan.	J Bacteriol.	193(24)	7010	2011
Kitao T, Tada T, Tanaka M, Narahara K, Shimojima M, Shimada K, Miyoshi-Akiyama T, Kirikae T.	Emergence of a novel multidrug-resitant *Pseudomonas aeruginosa* strai producing IMP-type metallo-β-lactamases and AAC(6')-Iae in Japan.	Int J Antimicrob Agents.	3791	1-4	2011
Kirikae T, Mori-Yoshikawa N, Arakawa Y.	Isolation rates of multidrug-resistant *Pseudomonas aeruginosa* and Acinetobacter spp. at medical facilities in Japan	BMC Infect Diseases.			Submitted
YamaguchiY, ImamuraK, Sasao A, Murakami E, Arakawa Y, Kurosaki H	Metal Preference of Zn(II) and Co(II) for Dinuclear Metal Binding Site of IMP-1 Metallo-β-lactamase and Spectroscopic Properties of Co(II)-substituted IMP-1 with Mercaptoacetic Acid	Med. Chem. Commun.	2	720-725	2011
Yamaguchi Y, Ding S, Murakami E, Imamura K, Fuchigami S, Hashiguchi R, Yutani K, Mori H, Suzuki S, Arakawa A, Kurosaki H	A Demetallation Method for IMP-1 Metallo-β-lactamase That Has Restored Enzymatic Activity Upon Addition of Metal Ion(s).	ChemBioChem	12	1979-1983	2011

Yamaguchi T. Nakamura I, Chiba K, Matsumoto T.	Epidemiological and Microbiological Analysis of Community-associated Methicillin-resistant *Staphylococcus aureus* Strains Isolated From a Japanese Hospital.	Jap. J. Infect. Dis.	in print		2012
Takahiro Tabuchi, Toshio Takatorige, Yukio Hirayama, Nobuaki Nakata, Shigeyoshi Harihara, Akira Shimouchi, Koshiro Fujita, Hiroko Yoshida, Yoshitaka Tamura, Takayuki Nagai, Tomoshige Matsumoto, Tetuya Takashima, Hiryoyasu Ito	Tuberculosis infection among homeless persons and caregivers in a high-tuberculosis-prevalence area in Japan: a cross-sectional study	BMC Infectious Diseases	11	22	2011
Tomohiro Oishi, Akihito Wada, Bin Chang, Shinichi Toyabe, and Makoto Uchiyama	Serotyping and multilocus sequence typing of *Streptococcus pneumoniae* isolates from the blood and posterior nares of Japanese children prior to the introduction of 7-Valent pneumococcal conjugate vaccine	Japanese Journal of Infectious Diseases	64	341-344	2011
Wachino J, Yamaguchi Y, Mori S, YamagataY, ArakawaY, Shibayama K.	Crystallization and Preliminary X-ray Analysis of the Subclass B3 Metallo-β-Lactamase SMB-1 that Confers Carbapenem Resistance.	Acta Crystallographica Section F	in press		2012

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
藤本 修平	「antibiogram の自動分類と二次元キャリアマップ(2DCM)」 による院内感染対策		IASR			2011	9-10
稲益　智子 出口　弘 市川　学 藤本　修平 齋藤　智也	伊豆大島における インフルエンザ様疾患 による島民の受診行動 (電子化システムの１つの方向であるシミュレーションの研究を行った中で、感染の動態に関する基礎的なデータが圧倒的に不足しているため伊豆大島をフィールドにインフルエンザの感染動態を調査したものです。東京工業大学、慶応大学との共同研究です。感染に関わるデータはEIDに投稿中です。参考まで。)		島しょ医療 研究会誌			2011	16-23

Ⅳ.　研究成果の刊行物・別刷・資料

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Nosocomial spread of multidrug-resistant group B streptococci with reduced penicillin susceptibility belonging to clonal complex 1

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Background: Multiple group B *Streptococcus* (GBS) isolates with reduced penicillin susceptibility (PRGBS) were recovered from several patients, hence a probable nosocomial transmission of PRGBS in a hospital setting was suspected.

Methods: Ten PRGBS recovered from eight patients in a general hospital were characterized. Sequence analysis of genes for penicillin-binding proteins (PBPs) and quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB* and *parC* was performed, and the macrolide resistance genes were detected by PCR. Genetic relatedness among the isolates was examined by PFGE and multilocus sequence typing.

Results: All the PRGBS had the key amino acid substitution V405A, together with F395L, R433H, H438Y and G648A in PBP 2X and T567I in PBP 2B. A 23S rRNA methylase gene, *erm*(B), was also found in all 10 PRGBS strains. PFGE analysis revealed considerable genetic relatedness among the isolates. Isolates of pulsotype I were obtained from four patients in ward A and one patient in ward B, while isolates of pulsotypes II and III were obtained from two patients in ward B and one patient in ward C, respectively. Isolates of pulsotype I were resistant to levofloxacin (MIC >8 mg/L) and had the following amino acid substitutions in the QRDRs: S81L in GyrA, E476K in GyrB and S79Y in ParC. However, pulsotype II strains resistant to levofloxacin (MIC 8 mg/L) had no change in GyrA, but changes in GyrB (E476K) and ParC (S79Y). All 10 PRGBS strains belonged to serotype VI and ST458 (where ST stands for sequence type).

Conclusions: This is the first description of the nosocomial spread of multidrug-resistant PRGBS strains belonging to the genetic lineage ST458.

Keywords: horizontal transmission, β-lactams, macrolides, fluoroquinolones, group B *Streptococcus*

Introduction

Group B *Streptococcus* (GBS) is one of the most important causes of serious neonatal infections. In particular, for early onset neonatal diseases, rectal or vaginal GBS colonization found in about 25% of pregnant women is the primary risk factor. GBS also causes invasive infections in adults, including pregnant women, elderly individuals and immunocompromised patients. Penicillin is the first-line antibiotic for treatment of GBS infection, as well as for intrapartum antibiotic prophylaxis to prevent early onset infection, because resistance to this agent has not been reported so far among GBS clinical isolates. However, in 2008 we reported GBS clinical isolates with reduced penicillin susceptibility (PRGBS),[1,2] in which an increase was noted in the MICs of β-lactam antibiotics including penicillin (MICs of 0.25–1 mg/L). Since no typical phenotypic changes are observed between GBS and PRGBS, it is very difficult to distinguish them in routine microbiology tests. However, elevation of ceftizoxime and ceftibuten MICs would be a good marker for screening for PRGBS,[1,3] although these agents are not included in the list of drugs for antimicrobial susceptibility testing. Amino acid substitutions V405A and Q557E in penicillin-binding protein (PBP) 2X, which are shared by most PRGBS strains, have been demonstrated as a major mechanism involved in reduction of GBS penicillin

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susceptibility.[1] Besides these two key substitutions that have been identified in PBP 2X, multiple amino acid substitutions were also found in PBPs 2X, 2B and 1A among PRGBS strains depending on their penicillin MIC levels. However, it has also been noted that one of those PRGBS strains had no such key substitution in PBP 2X.[1]

After our aforementioned study, GBS strains isolated in the USA, showing elevated MICs, but which were still susceptible to β-lactam antibiotics, shared amino acid substitution Q557E in PBP 2X,[4] whereas GBS strains with penicillin MICs of 0.25 or 0.5 mg/L from two Canadian studies had amino acid substitutions in several PBPs, but no key substitutions—V405A or Q557E—were found in PBP 2X.[5,6] Interestingly, the amino acid substitutions identified in PBPs of these Canadian PRGBS strains have not been found so far among the strains tested in our studies. Thus characterization of molecular mechanisms underlying the reduced penicillin susceptibility profile in PRGBS is still in progress.

Clindamycin or erythromycin is traditionally used for GBS intrapartum prophylaxis for penicillin-allergic women at high risk of anaphylaxis. However, increasing resistance of GBS to clindamycin or erythromycin has been reported worldwide. In the USA, the rates of resistance among invasive GBS isolates were 13%–15% for clindamycin and 26%–32% for erythromycin.[7,8] The Japan Nosocomial Infections Surveillance (JANIS) of the Ministry of Health, Labour and Welfare showed that the prevalence of resistance was 22% and 28% for clindamycin and erythromycin, respectively, among GBS from various clinical sources in 2010.[9] In GBS isolates, macrolide resistance has been mediated mainly by two classes of resistance genes: the *erm* genes, including *erm*(B), *erm*(TR)/*erm*(A) and *erm*(C), which mediate ribosomal methylation; and the *mef* genes, such as *mef*(A) and *mef*(E), which are involved with efflux of macrolides as they encode membrane-associated transporters. The *erm* genes are associated with the macrolide-lincosamide-streptogramin B resistance phenotype, which usually show cross-resistance to clindamycin, while *mef* genes are the resistance determinants specific for 14- and 15-membered macrolides.

Fluoroquinolone resistance in GBS has recently emerged in several countries including Japan.[10–14] Mutations in the quinolone resistance-determining regions (QRDRs) of the genes encoding topoisomerase IV ParC and DNA gyrase GyrA have mainly been associated with fluoroquinolone resistance of this organism.[10,11,13,15] The presence of GyrA-ParC-ParE triple mutations has recently been reported in Taiwan isolates.[14] High rates of tetracycline resistance have also been noted among GBS isolates with the most common resistance determinant, *tet*(M), which encodes the ribosomal protection protein.[16,17]

Our phylogenetic comparative analyses have shown genetic diversity of *pbp* genes among PRGBS strains, while those genes of the penicillin-susceptible strains were highly conserved, irrespective of their isolation dates.[2] Furthermore, a phylogenic tree showed three distinct genetic lineages of PRGBS strains, implying that those lineages have been independently emerging through the accumulation of different genetic mutations in their *pbp* genes during evolution. PRGBS has been found to be capable of surviving persistently at the site of infection for >3 weeks.[18]

In the present study we investigated the molecular basis of resistance determinants and the clonal relationship of 10 PRGBS isolates showing multidrug resistance to macrolides, lincosamides, fluoroquinolones and tetracyclines detected from eight patients during a 5 month period to characterize the genetic background of the isolates.

Materials and methods

Bacterial strains

Ten GBS clinical strains isolated from eight patients admitted to a general hospital located in Tokyo, Japan, during March–August 2007 were analysed. These strains included two strains, strain numbers 2-1 and 2-2, isolated from different specimens obtained at approximately the same time from one patient and two strains, strain numbers 4-1 and 4-2, isolated at 2 month intervals from another patient. The source of strains and the clinical backgrounds of the patients are shown in Table 1.

GBS strains were grown overnight in Todd–Hewitt broth (BD Diagnostics) and then stored in glycerol at −80°C until use.

Serotyping was performed using antisera (Denka Seiken, Tokyo, Japan) to the type-specific capsular polysaccharides Ia, Ib, II, III, IV, V, VI and VIII.

Antimicrobial susceptibility testing

MICs were determined using a broth microdilution method with a MicroScan MICroFAST panel type 5J system (Dade Behring Inc., Tokyo, Japan) by following the guidelines recommended by the CLSI.[19] For susceptibility categories we referred to the CLSI criteria.[20] Additionally, MICs of penicillin and ceftizoxime were determined by Etest according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

MIC determinations were repeated independently three times for each strain to ensure the reproducibility of the MICs by using quality control strain *Streptococcus pneumoniae* ATCC 49619.

Susceptibility testing with a ceftibuten disc was also performed by Kirby–Bauer's disc-diffusion method.[3] β-Lactamase activity was detected by a nitrocefin-based disc procedure (BD Diagnostics).

Analysis of pbp gene sequences

PCR amplification and sequencing analysis of each *pbp* gene were performed as previously described, with minor modifications.[2] Briefly, the full-length coding regions of *pbp1a*, *pbp2b* and *pbp2x* genes were amplified from genomic DNA extract using the primer pairs f1 and r1, as listed in Table 2. PCRs were carried out using PrimeSTAR HS DNA polymerase (Takara Shuzo Co., Kyoto, Japan) with reaction conditions of 30 cycles of 98°C for 10 s, 55°C for 5 s and 72°C for 2.5 min.

Sequencing analyses of both strands of purified PCR products were performed using several internal forward and reverse sequencing primers (Table 2), a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI 3730x/DNA analyser (Applied Biosystems).

The nucleotide sequences obtained were assembled into contigs with BioEdit (version 5.0.9) software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), then aligned with ClustalW software.[21] *Streptococcus agalactiae* strains 2603 V/R (ATCC BAA-611; GenBank accession number NC004116) and NEM316 (ATCC 12403; GenBank accession number NC004368) were used as reference strains for comparative analysis.

Analysis of macrolide and fluoroquinolone resistance

PCR detection of *erm*(B), *erm*(TR) and *mef*(A/E), conferring resistance to macrolides or lincosamides, was performed with specific primers shown in Table 2 as described previously.[22,23] For three representative strains, the PCR products were subjected to sequence analyses to confirm the identity of amplification products.

Table 1. Clinical associations and microbiological profiles of PRGBS isolates

					Patient					Bacterial isolate																	
												MIC (mg/L)															
Strain no.	Date of admission	Date of isolation	Ward	Department	age (years)	sex	underlying diseases	prior therapy (within 3 months)	specimens	pulsotype	serotype	PEN (≤0.12)[a]	AMP (≤0.25)	CTM	CTX (≤0.5)	CRO (≤0.5)	CDN	FEP (≤0.5)	CFM	ZOX[b]	MEM (≤0.5)	ERY (≤0.25)	CLR (≤0.25)	CLI (≤0.25)	LVX (≤2)	TET (≤2)	VAN (≤1)
1	8 December 2006	17 April 2007	A	NS	86	F	multiple aneurysm	MEM, CIP	PHA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
2-1	20 March 2007	15 April 2007	A	NS	70	M	cerebral infarction	CAZ, SAM, ABK, CIP	pus	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
2-2		21 April 2007							PHA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
3	25 March 2007	24 April 2007	B	IM	87	F	acute pneumonia	SAM	PHA	II	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	8	>4	0.5
4-1	5 February 2007	13 March 2007	A	NS	78	F	hydrocephalus	CAZ, ABK, VAN	PHA	I	VI	0.25[b]	0.25	2	0.25	0.25	0.12	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
4-2		7 May 2007							TTA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
5	24 March 2007	25 April 2007	A	NS	52	M	pancreas cancer	CFZ, SAM	TTA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
6	18 July 2007	30 July 2007	B	IM	84	M	brain-stem infarction	SAM	TTA	II	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	8	>4	0.5
7	22 February 2007	25 April 2007	B	IM	82	F	cerebral infarction	SAM, CIP, CTM, CFP/SUL	PHA	I	VI	0.25	0.5	2	0.25	0.25	0.25	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5
8	31 August 2007	31 August 2007	C	IM	82	F	cerebral infarction sequelae	unknown	TTA	III	VI	0.25[b]	0.25	2	0.25	0.25	0.12	≤0.5	>1	>32	0.25	>1	>1	>1	>8	>4	0.5

NS, neurosurgery; IM, internal medicine; F, female; M, male; PHA, pharyngeal swab; TTA, transtracheal aspirate; MEM, meropenem; CIP, ciprofloxacin; CAZ, ceftazidime; SAM, ampicillin/sulbactam; ABK, arbekacin; VAN, vancomycin; CFZ, cefazolin; CTM, cefotiam; CFP/SUL, cefoperazone/sulbactam; PEN, penicillin; AMP, ampicillin; CTX, cefotaxime; CRO, ceftriaxone; CDN, cefditoren; FEP, cefepime; CFM, cefixime; ZOX, ceftizoxime; ERY, erythromycin; CLR, clarithromycin; CLI, clindamycin; LVX, levofloxacin; TET, tetracycline.

[a]The CLSI's susceptible MICs of each antimicrobial for GBS.

[b]MIC results of the Etest.

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Table 2. Oligonucleotide primers

Target gene	Usage			Sequence[a]	Amplicon size (bp)	Reference
pbp1a	PCR primers	forward	f1	5′-CGGAATTCATGGGATTTATTATCTTAGCTA-3′	2209	1
		reverse	r1	5′-ACGTCGACTTAATTACCGTTAGGTACTGTA-3′		
	sequencing primers	forward	f1b	5′-ACACCAAAGAAGAAATTCTTAC-3′		
			f2	5′-TAAAGCAAAAATCTACTTATCC-3′		
			f2b	5′-GTAGTGAGAAAATGGCAGCGGC-3′		
			f3	5′-GCCTACATGATGACGGATATGC-3′		
			f3b	5′-CAAAATTCTGGACAGTCAAGTC-3′		
		reverse	r2	5′-TCCAATCTGCACTGTATCCGCC-3′		
			r3	5′-TAGCTGCTTTAGTACCAGTACC-3′		
			r4	5′-CAGCGGCTTCAAGTGCTCTGAC-3′		
			r5	5′-TGACTTTACCATTAGTCGCATC-3′		
			r6	5′-TTTTATCTTGATACATCTGCTG-3′		
pbp2b	PCR primers	forward	f1	5′-CGGAATTCATGTTGAATCGTAAAAAAAGGT-3′	2062	
		reverse	r1	5′-ACGTCGACTTATTGTCCTGTGAACTGTGAA-3′		
	sequencing primers	forward	f1b	5′-TTCATCTCAGTCTATCAAAGAG-3′		
			f2	5′-CTATTTCTACAGAAAAGGCAGG-3′		
			f2b	5′-AGAAAGTATCTTGAAACAATAC-3′		
			f3	5′-CAACTCTAATGGAATCGTTCGG-3′		
			f3b	5′-TGGACAAACAGTTTCTACCTAC-3′		
		reverse	r2	5′-CTATCTTATTTAGTGTTTTAGG-3′		
			r3	5′-GATAGCCTCGATCAGTTAAAGC-3′		
			r4	5′-CATGATCATTTTTCAGACCAGC-3′		
			r5	5′-CTCGGTCATTCAGTGAATAGCC-3′		
			r6	5′-TAGCGCTCACTGGAACTGCAGC-3′		
pbp2x	PCR primers	forward	f1	5′-CGGAATTCGTGACTTTTTTTAAAAAGCTAA-3′	2275	
		reverse	r1	5′-ACGTCGACTTAATCTCCTATTGTAATTTTG-3′		
	sequencing primers	forward	f1b	5′-AACTATACGACAGCTACAGGTC-3′		
			f2	5′-GTAGTGGGAATGTTCTTTTAGG-3′		
			f2b	5′-TCTAAGCATTTTAACTCTACTG-3′		
			f3	5′-AAGAAGCAGCTAGTAAAACACG-3′		
			f3b	5′-GAAAATCCAGGTCATGTAGCGG-3′		
		reverse	r2	5′-GAACCAGATTACGACGTAATTC-3′		
			r3	5′-CAGATTTTACTGCAACTGATTG-3′		
			r4	5′-ATGAGCTCATAGCGATAGTTAC-3′		
			r5	5′-TTGCAGAGGCTAGAGTCATTAC-3′		
			r6	5′-CCGCCCTACGTTCTGTTGTTGC-3′		
			r7	5′-AAGACAATCCTGAACCTGAACTTCC-3′		
			r8	5′-TATCTGTACCAACGATGATGAC-3′		
erm(B)	PCR primers	forward	f1	5′-ATTGGAACAGGTAAAGGGC-3′	442	23
		reverse	r1	5′-GAACATCTGTGGTATGGCG-3′		
erm(TR)	PCR primers	forward	f1	5′-GAAGTTTAGCTTTCCTAA-3′	395	22
		reverse	r1	5′-GCTTCAGCACCTGTCTTAATTGAT-3′		
mef(A/E)	PCR primers	forward	f1	5′-AGTATCATTAATCACTAGTGC-3′	346	
		reverse	r1	5′-TTCTTCTGGTACTAAAAGTGG-3′		
gyrA	PCR primers	forward	f1	5′-GCCATGAGTGTCATTGTTGC-3′	599	in this study
		reverse	r1	5′-ATCACCAAGGCACCAGTAGG-3′		
gyrB	PCR primers	forward	f1	5′-TTTCGTACTGCCTTGACACG-3′	650	
		reverse	r1	5′-TCAACATCGGCATCAGTCAT-3′		
parC	PCR primers	forward	f1	5′-CGTTTTGGGCGCTATTCTAA-3′	607	
		reverse	r1	5′-TAGCGCCAGTTGGAAAATCT-3′		

[a]Restriction sites are underlined.

PCR amplification and DNA sequencing of the *gyrA*, *gyrB* and *parC* genes, which include the QRDRs responsible for the fluoroquinolone resistance phenotype, were performed using primers specifically designed from known DNA sequences of strain 2603 V/R as shown in Table 2. PCR and sequence analysis were carried out as described above except for an extension time of 1 min.

PFGE

PFGE of SmaI (Takara)-digested chromosomal DNAs was performed as previously described by Nagano *et al.*[2] Lambda DNA ladder (48.5 kb–1 Mb; Takara) was used as a molecular size marker. PFGE results were interpreted according to the Tenover's criteria.[24]

Multilocus sequence typing (MLST)

MLST was performed as described previously.[25] Amplification of seven housekeeping genes—*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*—by PCR was carried out using amplification primers and PrimeSTAR HS DNA polymerase (Takara) with reaction conditions of 1 cycle of 98°C for 1 min, followed by 30 cycles of 98°C for 10 s, 55°C for 15 s and 72°C for 1 min, and finally 1 cycle of 72°C for 7 min. PCR products were purified and sequenced using sequencing primers. Allelic profile assignment and sequence type (ST) determinations were made using the GBS MLST databases (http://pubmlst.org/sagalactiae). eBURST analysis was performed to define clonal complexes (CCs) within the isolates by using the eBURST program (http://eburst.mlst.net/v3/enter_data/single/).

Results

Origin of GBS strains

GBS strains were obtained from eight inpatients (five females and three males), aged 52–86 years (mean age 77.6 ± 11 years) (Table 1). Four patients were admitted to neurosurgery in ward A and the remaining four patients were admitted to internal medicine, among whom three were in ward B and one was in ward C. β-Lactams had been administered in all patients except one, whose history of prior therapy was unknown, and ciprofloxacin had been prescribed for three patients within 3 months of isolation of GBS. All GBS clinical strains showed no growth inhibitory zone around the ceftibuten disc, suggesting PRGBS.[3] Of those 10 isolates, 5 (strains 1, 2-2, 3, 4-1 and 7) were recovered from pharyngeal swab samples, 4 (strains 4-2, 5, 6 and 8) were from transtracheal aspirate samples and 1 (strain 2-1) was from pus obtained from the gastrostomy site. All strains were serotyped as type VI.

MICs

The MICs of several antimicrobial agents for *S. pneumoniae* ATCC 49619 were all within the quality control ranges defined by the CLSI,[20] and reproducible MIC results were obtained for GBS strains tested against all antimicrobials (Table 1).

The broth microdilution method showed that the MICs of penicillin and ampicillin were 0.25 mg/L and 0.5 mg/L, respectively, for eight strains, against 0.12 mg/L and 0.25 mg/L, respectively, for strains 4-1 and 8. However, reproducible results of a penicillin MIC of 0.25 mg/L were obtained by the Etest for these two strains. Thus all 10 strains were confirmed to be PRGBS. The MICs of cefotaxime, ceftriaxone and meropenem for PRGBS strains were all still within the susceptible range (≤0.5 mg/L), as shown in Table 1, but were higher than those for penicillin-susceptible strains possessing no amino acid substitutions in PBPs 2X, 2B or 1A.[2] The MICs of erythromycin, clarithromycin and clindamycin were >1 mg/L for all isolates tested, which fell into the resistant category. The 10 strains were resistant to levofloxacin, including MICs >8 mg/L for eight strains and 8 mg/L for strains 3 and 6. All strains were resistant to tetracycline (MIC >4 mg/L), but susceptible to vancomycin. β-Lactamase activity was not detected in any of the PRGBS isolates.

Table 3. Deduced amino acid substitutions in PBPs 2X, 2B and 1A

	Amino acid substitutions in	
	PRGBS	both PRGBS and PSGBS[a]
PBP2X	F395L, V405A, R433H, H438Y, G648A	I377V, V510I
PBP2B	T567I	—
PBP1A	—[b]	—

[a]Refers to Nagano *et al.*[2]
[b]No detected substitution.

Nucleotide sequences and amino acid substitutions in PBPs 2X, 2B and 1A

DNA sequencing of the *pbp* genes revealed that *pbp2x*, *pbp2b* and *pbp1a* genes of 10 PRGBS strains were identical. Those strains shared 10 nucleotide mutations, including five non-synonymous substitutions in *pbp2x* genes when compared with the corresponding genes of strains 2603 V/R and NEM316. The PBP 2X amino acid substitutions included a key substitution, V405A, and four additional substitutions—F395L, R433H, H438Y and G648A—that had been unique to PRGBS in our previous studies.[1,2] In PBP 2B, one amino acid substitution, T567I, which had been unique to PRGBS, was found. No amino acid substitutions were observed in PBP 1A (Table 3).

Macrolide and fluoroquinolone resistance determinants

Amplification of DNA from 10 PRGBS strains yielded PCR products of the expected sizes (442 bp) with *erm*(B)-specific primers. Sequence analyses of the PCR products from three representative strains—1, 3 and 8—selected on the basis of PFGE types revealed that the sequences of those amplified products were identical to the *erm*(B) gene sequence in GenBank accession number EF422361. All strains were negative for the *erm*(TR) and *mef*(A/E) genes.

Table 4 summarizes the QRDR amino acid substitutions in GyrA, GyrB and ParC of the PRGBS strains. Eight strains—1, 2-1, 2-2, 4-1, 4-2, 5, 7 and 8—for which levofloxacin MICs were >8 mg/L had an E476K substitution in GyrB in addition to S81L in GyrA and S79Y substitutions in ParC. The remaining strains—3 and 6—for which levofloxacin MICs were 8 mg/L, also had E476K in GyrB and S79Y in ParC, but had no substitutions in GyrA.

PFGE and MLST analyses

Figure 1 shows the PFGE results of SmaI-digested chromosomal DNAs from 10 PRGBS strains, representing three different PFGE

Table 4. Amino acid substitution in QRDRs of GyrA, GyrB and ParC, and MICs of levofloxacin

Strain	Pulsotype	MIC of levofloxacin (mg/L)	Amino acid substitution		
			GyrA	GyrB	ParC
1, 2-1, 2-2, 4-1, 4-2, 5, 7, 8	I, III	>8	S81L	E476K	S79Y
3, 6	II	8	—[a]	E476K	S79Y

[a]No detected mutation.

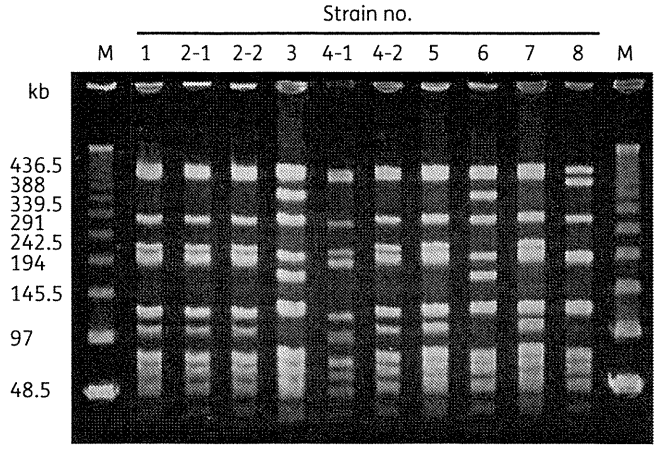

Figure 1. PFGE profiles of genomic DNA of multidrug-resistant PRGBS isolates digested with SmaI. M, bacteriophage lambda DNA ladder as molecular size markers.

patterns. The predominant PFGE type I included seven strains—1, 2-1, 2-2, 4-1, 4-2, 5 and 7—all of which were derived from ward A, except strain 7 from ward B. PFGE type II included strains 3 and 6 derived from ward B, and type III included strain 8 from ward C. PFGE types II and III differed from PFGE type I by four and three bands, respectively, and those three types were therefore considered to have a similar genetic background.

PFGE type I strains had the QRDR amino acid substitutions in GyrA, GyrB and ParC, but PFGE type II strains had those in only GyrB and ParC (Table 4).

All 10 PRGBS with the allelic profile 1, 1, 2, 1, 1, 2 and 3, in the order *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK* and *tkt*, were assigned to ST458, which is a single-locus variant of ST1 within CC1.

Discussion

This study describes a probable nosocomial spread of PRGBS caused by genetically very similar PRGBS isolates that acquired multidrug resistance to macrolides, lincosamides, fluoroquinolones and tetracyclines, as well as several oral cephalosporins such as ceftizoxime.[1,3]

Among 10 PRGBS isolates derived from eight patients, a close genetic relationship was strongly suggested by PFGE analysis. Four patients in ward A and one patient in ward B had strains with the predominant PFGE type (pulsotype) I, and these strains were isolated in the same period, indicating probable bacterial transmission between these wards. Transmission of PRGBS strains was also noted in ward B, where two patients were found to share the same strains with pulsotype II. A strain with pulsotype III was isolated from a patient in ward C on the day of her admission, so it was suggested that the strain might have been introduced into the hospital from the community or other medical settings. The isolation of PRGBS strains with pulsotype II was preceded by those with pulsotype I, which, initially, might lead one to assume that pulsotype II strains are derivatives of pulsotype I strains. However, pulsotype I strains had the QRDR amino acid substitutions in GyrA, GyrB and ParC, whereas pulsotype II strains had those only in GyrB and ParC, which contradicted our speculation. Thus it may well be that genetically related strains of pulsotypes I and II spread separately on a ward or between two wards. PRGBS strains 4-1 and 4-2 sharing pulsotype I were detected from a patient over a 2 month interval, suggesting those strains might colonize persistently, as has been observed previously.[18]

In eight PRGBS strains of pulsotypes I and III with levofloxacin MICs >8 mg/L, E476K in GyrB was newly detected, together with two substitutions S81L in GyrA and S79Y in ParC, which have been found to be involved with high-level fluoroquinolone resistance. The remaining two strains of pulsotype II for which levofloxacin MICs were 8 mg/L also had E476K in GyrB and S79Y in ParC, but had no substitutions in GyrA. To the best of our knowledge, although amino acid substitutions in GyrB have not been reported in GBS, E476K, which corresponds to the E474K substitution in *S. pneumoniae*, may possibly contribute to fluoroquinolone resistance, as has been suggested for *S. pneumoniae*.[26]

Clindamycin can be used as intrapartum GBS prophylaxis for penicillin-allergic GBS carriers, as has been endorsed by the CDC.[27] All PRGBS strains in this study showing consistent resistance to macrolides and lincosamides harboured *erm*(B) genes that confer high resistance levels to these agents. This finding enhances the need for monitoring of GBS strains multiresistant to macrolides and lincosamides. Those strains were also resistant to tetracycline, and erythromycin resistance genes have sometimes been found on the mobile genetic elements encoding tetracycline resistance genes.[28,29]

The nucleotide sequences of the coding regions of *pbp2x*, *pbp2b* and *pbp1a* genes were completely identical in all 10 PRGBS, although the strains were divided into three genetically related PFGE types. Five amino acid substitutions, including a key substitution (V405A) identified in PBP 2X, have been found to be unique to PRGBS.[1,2] In PBP 2B, one amino acid substitution that is also unique to PRGBS was found. It is of interest that those sequences found in *pbp2x*, *pbp2b* and *pbp1a* genes were 100% identical to the corresponding sequences of the PRGBS strain R5, which has been reported previously.[2] Moreover, the deduced amino acid sequences of PBPs 2X, 2B and 1A were completely identical to the corresponding sequences of the previously described PRGBS strains R1, R2, R5 and R6, which has one additional substitution in PBP 2X. Ten PRGBS strains characterized in the present study and PRGBS strains R1, R2, R5 and R6, which have been found to form one of the three distinct lineages of the *pbp* genes in our previous study,[2] were all serotype VI and had closely related PFGE profiles. MLST analysis

showed that all 10 PRGBS were ST458, which is a single-locus variant of ST1 belonging to CC1. Interestingly, PRGBS strains R1, R2, R5 and R6 were serotype VI and were also assigned to ST458, which has been reported to be the predominant ST among PRGBS in Japan.[30] Thus the spread of a clonal serotype VI PRGBS population of ST458 has occurred in geographically separate areas, while acquiring resistance to macrolides, lincosamides and fluoroquinolones over time. ST458, which has been assigned as a new ST to our PRGBS strains, has not been detected among PRGBS strains from other countries.[4,6] The serotype VI PRGBS with ST458 may well be defined as the Japan clone.

EUCAST (http://www.eucast.org/clinical_breakpoints/) has established a clinical breakpoint for penicillin and *Streptococcus* groups A, B, C and G, including the resistance criteria of penicillin MIC >0.25 mg/L. However, EUCAST notes that the strains with MIC values above the epidemiological cut-off value (0.25 mg/L) are very rare or not yet reported, and they should be reported as 'resistant' until there is evidence regarding clinical response for confirmed isolates with MIC above the current resistant breakpoint. According to CLSI M100-S21,[20] only susceptible interpretive criteria are available for penicillin and β-haemolytic streptococci, with several comments, e.g. non-susceptible isolates with penicillin MICs >0.12 mg/L and ampicillin MICs >0.25 mg/L are extremely rare in β-haemolytic streptococci and have not been reported for group A *Streptococcus*. CLSI also states that susceptibility testing of penicillins and other β-lactams for the treatment of β-haemolytic streptococcal infections need not be performed routinely. However, in Canada, the development of penicillin non-susceptibility in GBS has been described *in vivo* in adult patients by acquiring amino acid substitutions in PBPs during prolonged administration of penicillin V.[5,6] These findings underscore the necessity of routinely monitoring the levels of penicillin MICs, especially when penicillins or cephalosporins are prescribed to a patient over a long period. Moreover, in the present study we have demonstrated probable horizontal transmission of PRGBS strains among patients, leading to their nosocomial spread for at least 5 months. The clinical significance of PRGBS isolates in antimicrobial chemotherapy and also in intrapartum prophylaxis remains unclear. Thus investigation and discussion should be encouraged to predict the therapeutic effect of penicillin therapy, especially for more invasive GBS infections such as meningitis due to PRGBS.

Our findings extend the knowledge about PRGBS with regard to more serious therapeutic and prophylactic problems posed by the possible future prevalence of multidrug-resistant genotypes of PRGBS, together with their ability to spread and survive in hospital environments. The emergence of multidrug-resistant PRGBS is a concern regarding future global spread, as we have experienced with multidrug-resistant *S. pneumoniae*,[31,32] and again emphasizes the need for careful epidemiological monitoring of GBS strains to assess the current prevalence status of PRGBS as well as their multidrug-resistant genotypes.

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Transparency declarations

None to declare.

References

1 Kimura K, Suzuki S, Wachino J *et al.* First molecular characterization of group B streptococci with reduced penicillin susceptibility. *Antimicrob Agents Chemother* 2008; **52**: 2890–7.

2 Nagano N, Nagano Y, Kimura K *et al.* Genetic heterogeneity in *pbp* genes among clinically isolated group B streptococci with reduced penicillin susceptibility. *Antimicrob Agents Chemother* 2008; **52**: 4258–67.

3 Kimura K, Wachino J, Kurokawa H *et al.* Practical disk diffusion test for detecting group B *Streptococcus* with reduced penicillin susceptibility. *J Clin Microbiol* 2009; **47**: 4154–7.

4 Dahesh S, Hensler ME, Van Sorge NM *et al.* Point mutation in the group B streptococcal *pbp2x* gene conferring decreased susceptibility to β-lactam antibiotics. *Antimicrob Agents Chemother* 2008; **52**: 2915–8.

5 Gaudreau C, Lecours R, Ismaïl J *et al.* Prosthetic hip joint infection with a *Streptococcus agalactiae* isolate not susceptible to penicillin G and ceftriaxone. *J Antimicrob Chemother* 2010; **65**: 594–5.

6 Longtin J, Vermeiren C, Shahinas D *et al.* Novel mutations in a patient isolate of *Streptococcus agalactiae* with reduced penicillin susceptibility emerging after long term oral suppressive therapy. *Antimicrob Agents Chemother* 2011; **55**: 2983–5.

7 Castor ML, Whitney CG, Como-Sabetti K. Antibiotic resistance patterns in invasive group B streptococcal isolates. *Infect Dis Obstet Gynecol* 2008; **2008**: 727505.

8 Phares CR, Lynfield R, Farley MM *et al.* Epidemiology of invasive group B streptococcal disease in the United States, 1999–2005. *JAMA* 2008; **299**: 2056–65.

9 Ministry of Health Labour and Welfare. *The Japan Nosocomial Infections Surveillance*. http://www.nih-janis.jp/ (2 September 2011, date last accessed).

10 Biedenbach DJ, Toleman MA, Walsh TR *et al.* Characterization of fluoroquinolone-resistant beta-hemolytic *Streptococcus* spp. isolated in North America and Europe including the first report of fluoroquinolone-resistant *Streptococcus dysgalactiae* subspecies *equisimilis*: report from the SENTRY Antimicrobial Surveillance Program (1997–2004). *Diagn Microbiol Infect Dis* 2006; **55**: 119–27.

11 Kawamura Y, Fujiwara H, Mishima N *et al.* First *Streptococcus agalactiae* isolates highly resistant to quinolones, with point mutations in *gyrA* and *parC*. *Antimicrob Agents Chemother* 2003; **47**: 3605–9.

12 Miro E, Rebollo M, Rivera A *et al.* *Streptococcus agalactiae* highly resistant to fluoroquinolones. *Enferm Infecc Microbiol Clin* 2006; **24**: 562–3.

13 Wehbeh W, Rojas-Diaz R, Li X *et al.* Fluoroquinolone-resistant *Streptococcus agalactiae*: epidemiology and mechanism of resistance. *Antimicrob Agents Chemother* 2005; **49**: 2495–7.

14 Wu H, Janapatla R, Ho Y *et al.* Emergence of fluoroquinolone resistance in group B streptococcal isolates in Taiwan. *Antimicrob Agents Chemother* 2008; **52**: 1888–90.

15 Murayama SY, Seki T, Sakata H *et al.* Capsular type and antibiotic resistance in *Streptococcus agalactiae* isolates from patients, ranging from newborns to the elderly, with invasive infections. *Antimicrob Agents Chemother* 2009; **53**: 2650–3.

16 Granlund M, Axemo P, Bremme K *et al.* Antimicrobial resistance in colonizing group B Streptococci before the implementation of a Swedish intrapartum antibiotic prophylaxis program. *Eur J Clin Microbiol Infect Dis* 2010; **29**: 1195–201.

17 Sadowy E, Matynia B, Hryniewicz W. Population structure, virulence factors and resistance determinants of invasive, non-invasive and colonizing *Streptococcus agalactiae* in Poland. *J Antimicrob Chemother* 2010; **65**: 1907–14.

18 Nagano N, Kimura K, Nagano Y *et al.* Molecular characterization of group B streptococci with reduced penicillin susceptibility recurrently isolated from a sacral decubitus ulcer. *J Antimicrob Chemother* 2009; **64**: 1326–8.

19 Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically—Eighth Edition: Approved Standard M7-A8*. CLSI, Wayne, PA, USA, 2009.

20 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-First Informational Supplement M100-S21*. CLSI, Wayne, PA, USA, 2011.

21 Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994; **22**: 4673–80.

22 Gigax SE, Schuyler JA, Kimmel LE *et al.* Erythromycin and clindamycin resistance in group B streptococcal clinical isolates. *Antimicrob Agents Chemother* 2006; **50**: 1875–7.

23 Marimón JM, Valiente A, Ercibengoa M *et al.* Erythromycin resistance and genetic elements carrying macrolide efflux genes in *Streptococcus agalactiae*. *Antimicrob Agents Chemother* 2005; **49**: 5069–74.

24 Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; **33**: 2233–9.

25 Jones N, Bohnsack JF, Takahashi S *et al.* Multilocus sequence typing system for group B *Streptococcus*. *J Clin Microbiol* 2003; **41**: 2530–6.

26 Weigel LM, Anderson GJ, Facklam RR. Genetic analyses of mutations contributing to fluoroquinolone resistance in clinical isolates of *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 2001; **45**: 3517–23.

27 Verani JR, McGee L, Schrag SJ. Prevention of perinatal group B streptococcal disease: revised guidelines from CDC. *MMWR Recommend Rep* 2010; **59** (RR-10): 1–32.

28 Brenciani A, Bacciaglia A, Vecchi M *et al.* Genetic elements carrying *erm*(B) in *Streptococcus pyogenes*, and association with *tet*(M) tetracycline resistance gene. *Antimicrob Agents Chemother* 2007; **51**: 1209–16.

29 Varaldo PE, Montanari MP, Giovanetti E. Genetic elements responsible for erythromycin resistance in streptococci. *Antimicrob Agents Chemother* 2009; **53**: 343–53.

30 Kimura K, Nagano N, Nagano Y *et al.* Predominance of sequence type 1 group with serotype VI among group B streptococci with reduced penicillin susceptibility identified in Japan. *J Antimicrob Chemother* 2011; **66**: 2460–4.

31 Appelbaum PC. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. *Clin Infect Dis* 1992; **15**: 77–83.

32 Reinert RR. The antimicrobial resistance profile of *Streptococcus pneumoniae*. *Clin Microbiol Infect* 2009; **15** Suppl 3: 7–11.

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Predominance of sequence type 1 group with serotype VI among group B streptococci with reduced penicillin susceptibility identified in Japan

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Background: Although group B *Streptococcus* (GBS; i.e. *Streptococcus agalactiae*) has been considered to be uniformly susceptible to β-lactams, GBS isolates with reduced penicillin susceptibility (PRGBS) have been reported from Japan and North America. In this study, PRGBS from Japan were characterized by multilocus sequence typing (MLST) and the results compared with data on PRGBS reported from the USA.

Methods: Twenty-eight clinical isolates of PRGBS recovered in Japan (including 22 isolates previously analysed by PFGE) were analysed by MLST and eBURST (http://eburst.mlst.net/).

Results: Twenty-three isolates were found to belong to the sequence type 1 (ST1) group (11 ST458, 7 ST1, 3 ST297, 1 ST358 and 1 ST4), while the remaining 5 isolates formed the ST23 group. Among 11 ST458 and 7 ST1 isolates, 9 and 4 were serotype VI, respectively, indicating a probable correlation between the ST1 group and serotype VI for PRGBS in Japan.

Conclusions: PRGBS in Japan could be classified into at least two ST groups, ST1 and ST23, which are genetically different from the ST19 PRGBS isolated in the USA, though five allele variations were seen between ST1 and ST19, implying a slight genetic relatedness.

Keywords: β-lactams, non-susceptible, GBS, multilocus sequence typing

Introduction

Group B *Streptococcus* (GBS; i.e. *Streptococcus agalactiae*) is a major cause of neonatal sepsis and meningitis, and also an important pathogen for elderly people and those suffering from underlying medical disorders.[1–5] Invasive infections caused by GBS in neonates (including very low birth weight infants) are associated with high mortality and morbidity.[3,6–8] About 5% of GBS-infected infants die, and if they survive they often suffer from severe neurological sequelae such as mental retardation and visual and/or auditory disabilities.[6] Penicillin generally remains the first-line agent for the treatment of GBS infections, as most strains remain susceptible.[6,9] However, we recently identified and molecularly characterized several clinical GBS isolates demonstrating reduced penicillin susceptibility (PRGBS) through acquisition of multiple mutations in the penicillin-binding protein 2X (PBP2X) gene.[10–12] PRGBS was also identified subsequently by several groups in the USA,[13] Canada[14] and Japan.[15] Previously we reported that all but two PRGBS isolates from Japan showed different banding patterns when analysed by PFGE using the ApaI restriction endonuclease.[10,11] In contrast, multilocus sequence typing (MLST) showed that each of four isolates in the USA belonged to the same sequence type (ST), namely ST19.[13] Since it is not well investigated whether or not PRGBS belong to a specific genetic lineage, we determined the ST of 28 PRGBSs isolated in Japan, including 22 isolates previously analysed by PFGE using ApaI digestion.[10,11]

Materials and methods

The PRGBS isolates were mostly from respiratory specimens of elderly people and one strain, MRY08-1422, was from blood (Table 1). Chromosomal DNA was prepared using the Wizard genomic DNA purification kit (Promega) and MLST was performed with minor modifications as described previously.[16] Amplifications of partial loci of seven housekeeping genes established by Jones *et al.*[16] and sequence analyses were performed as

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Table 1. STs and characteristics of the 28 PRGBS strains

Strain	ST	Serotype	Specimen	Year of isolation	Reference
B1	4[a]	III	sputum	1995	10
B6	1	VIII	sputum	1997	10
B7	23	III	sputum	1997	10
B8	458[a]	VI	sputum	1997	10
B10	297[a]	III	sputum	1997	10
B12	297[a]	III	sputum	1997	10
B40	297[a]	III	sputum	1997	10
B60	23	III	sputum	1998	10
B68	1	VI	sputum	1998	10
B502	458[a]	VI	sputum	2005	10
B503	458[a]	Ib	sputum	2005	10
B513	1	III	sputum	2005	10
B514	458[a]	VI	sputum	2005	10
B516	458[a]	III	sputum	2005	10
MRY06-238	1	VI	sputum	2006	this study
MRY06-241	458[a]	VI	sputum	2006	this study
MRY08-517	1	VI	sputum	2005	this study
MRY08-527	358[a]	Ib	sputum	2008	this study
MRY08-528	458[a]	VI	TTA	2005	this study
MRY08-1422	464	III	blood	2008	this study
R1	458[a]	VI	TTA	2003	11
R2	458[a]	VI	TTA	2003	11
R3	1	Ib	CS	2004	11
R4	1	VI	sputum	2004	11
R5	458[a]	VI	PHA	2003	11
R6	458[a]	VI	sputum	2004	11
R7	23	Ia	sputum	2004	11
R8	23	NT	sputum	2004	11

TTA, transtracheal aspirate; CS, conjunctival sac discharge; PHA, pharyngeal swab; NT, non-typeable.
[a]This ST belongs to the 'ST1 group', as it possesses no more than three allelic changes compared with ST1.

described previously.[10] After allelic profiling, the ST was assigned through the MLST website for *S. agalactiae* (http://pubmlst.org/sagalactiae). eBURST analysis was performed using eBURST version 3, available through the website http://eburst.mlst.net/.

Results

The STs and characteristics of the 28 PRGBSs are listed in Table 1. All were exactly confirmed as PRGBS by determination of the MIC of penicillin by the agar dilution method as recommended by the CLSI and sequence analysis of the PBP2X gene. The results were consistent with the results of the disc diffusion tests using ceftibuten discs.[17] The MICs of penicillin for the 28 isolates were in the range 0.25–1 mg/L. Among the 28 clinical isolates, 26 harboured both or either of the two PRGBS-specific amino acid substitutions, Q557E and V405A, in PBP2X. Although the two remaining clinical isolates, B7 and MRY08-1422, harboured neither the Q557E nor V405A substitution in PBP2X, these clinical isolates harboured several amino acid substitutions other than Q557E and V405A in the transpeptidase domain of PBP2X, although the contribution of those substitutions to the augmented MICs of several β-lactams remains to be elucidated.

Table 2. STs, ST profiles, allelic profiles and numbers of PRGBSs

ST	ST profile	Allelic profile (*adhP, pheS, atr, glnA, sdhA, glcK, tkt*)	Number of PRGBSs (%)
ST1	ST1	1, 1, 2, 1, 1, 2, 2	7 (25)
ST458[a]	one allelic variant of ST1	1, 1, 2, 1, 1, 2, 3	11 (39)
ST297[a]	one allelic variant of ST1	1, 1, 2, 2, 1, 2, 2	3 (11)
ST4[a]	three allelic variant of ST1	1, 1, 4, 1, 1, 3, 4	1 (4)
ST358[a]	three allelic variant of ST1	1, 1, 4, 1, 3, 3, 2	1 (4)
ST23	ST23	5, 4, 6, 3, 2, 1, 3	4 (14)
ST464	three allelic variant of ST23	5, 4, 4, 3, 2, 3, 1	1 (4)
			total=28 (100)

[a]This ST belongs to the 'ST1 group', as it possesses no more than three allelic changes compared with ST1 (see the 'Allelic profile' column).

The STs of the 28 isolates are shown in Tables 1 and 2. Eleven of the 28 isolates (39%) belonged to ST458, a new ST identified in the present study. The numbers of PRGBS isolates of ST1, ST23 and ST297 were 7 (25%), 4 (14%) and 3 (11%), respectively. The remaining three isolates belonged to ST4, ST358 and ST464.

By eBURST analysis, a single allelic difference was seen in both ST458 (allelic profile: 1, 1, 2, 1, 1, 2, 3) and ST297 (allelic profile: 1, 1, 2, 2, 1, 2, 2) when compared with ST1 (allelic profile: 1, 1, 2, 1, 1, 2, 2), therefore ST1, ST458 and ST297 formed clonal complex (CC) 1, as illustrated in Figure 1. There were 21 isolates of ST1, ST458 and ST297, accounting for 75% of the PRGBS tested. Thus CC1 was the predominant CC of PRGBS characterized in the present study. Moreover, ST4 (allelic profile: 1, 1, 4, 1, 1, 3, 4) and ST358 (allelic profile: 1, 1, 4, 1, 3, 3, 2) have three allelic differences compared with ST1 (allelic profile: 1, 1, 2, 1, 1, 2, 2); these two STs also belong to CC1 and form the ST1 group, together with ST1, ST458 and ST297. On the other hand, ST23 (four isolates) and ST464 (one isolate) belonged to a group far different from the ST1/ST19 group (Figure 1a and Figure S1). ST464 (allelic profile: 5, 4, 4, 3, 2, 3, 1) has three allelic differences compared with ST23 (allelic profile: 5, 4, 6, 3, 2, 1, 3) and forms the ST23 group (Figure 1b and Figure S1). Interestingly, ST17 was not found in the PRGBS from this study, despite the fact that ST17 has been reported as the most frequent ST among the GBS recovered from neonatal meningitis.[18–22]

Discussion

PRGBSs have thus far been mainly isolated from respiratory specimens of elderly people in Japan. In this study the STs of 28 PRGBSs were found predominantly to belong to the ST1 group, with a minority of isolates belonging to the ST23 group. ST1 and ST23 were reported as the major STs involved in carriage and invasive infections in neonates and non-pregnant adults,[16,23,24] and one strain, MRY08-1422, isolated from blood was assigned to ST464, and thus belonged to the ST23 group. Both ST1 and ST23 have been frequently identified among the isolates of throat flora.[25]

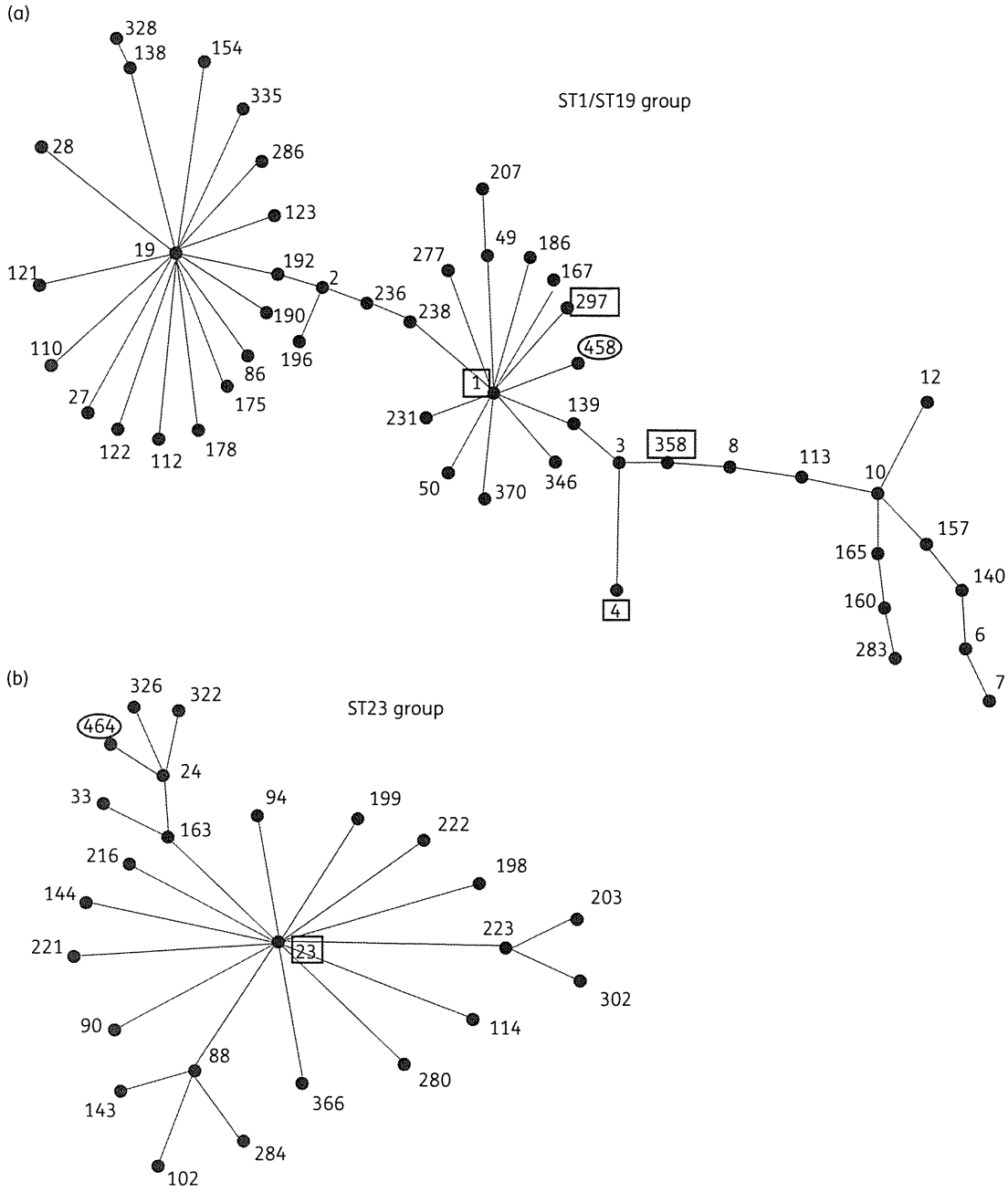

Figure 1. Rough sketch of eBURST analysis of STs of PRGBS. (a) ST1/ST19 group. (b) ST23 group. Numbers in the figure indicate STs. Numbers in squares or ellipses indicate STs of PRGBS. Numbers in ellipses indicate novel STs of PRGBS. Numbers that are not in squares or circles are STs of GBS other than the PRGBS in this study. The eBURST analysis connected two STs with one allelic profile difference by one line. The lengths of lines between two STs do not reflect the genetic distances between two STs. This rough sketch was created with random deletion of STs of GBS other than PRGBS from Figure S1 (available as Supplementary data at *JAC* Online) in order to simplify the original sketch. Therefore, this rough sketch does not contain all of the STs of GBS.

Although information concerning STs isolated from respiratory specimens of elderly people is limited, the STs of PRGBSs determined in this study might reflect the fact that most PRGBSs isolated so far have tended to be from respiratory specimens of elderly people.

The most frequent ST of PRGBS found in the present study was the novel type ST458. The eBURST analysis showed that ST458 is a single allelic variant of ST1. Interestingly, among the 11 ST458 and 7 ST1 strains, 9 and 4 were serotype VI, respectively (Table 1), suggesting a probable correlation between the ST1 group and

serotype VI in the PRGBSs tested. Limited information about the correlation between serotype VI and ST is available in GBS at present, but the ST of three serotype VI GBS strains deposited in the PubMLST database (http://pubmlst.org/sagalactiae/) are all ST1. Moreover, among six GBS strains with serotype VI, four strains were reported to be ST1 and the remaining two strains were ST14 (one allelic variant of ST1) and ST13 (five allelic variant of ST1), respectively.[16] Although ST458 clinical isolates with serotype VI may have biological characteristics similar to those of ST1, more information about the strains of ST458 will be needed to evaluate the correlation between serotype VI and the ST1-group PRGBS, together with its clinical significance.

As we reported previously, the PFGE analysis of PRGBS using ApaI showed different band patterns among the PRGBS clinical isolates tested. Moreover, phylogenetic analyses of the PBP genes of PRGBS suggested their genetically divergent origin.[10,11] However, an American study reported that four PRGBSs isolated in different states all belonged to ST19, suggesting a clonal expansion of PRGBS in the USA.[13] In the present study, however, ST19 was not found among the 28 PRGBSs isolated in Japan, and a greater variety of STs was observed among the PRGBS strains than in those isolated in the USA. We confirmed, therefore, that PRGBS in Japan could be classified into at least two ST groups, the ST1 group and the ST23 group, which are genetically different from the ST19 PRGBS isolated in the USA. However, two allelic similarities are found between ST1 and ST19, and these groups may well form the ST1/ST19 group as shown in Figure 1(a). This observation might suggest that the strains belonging to the ST1/ST19 group tend to have an ability to develop reduced penicillin susceptibility among various STs in GBS.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Baker CJ. Group B streptococcal infections. In: Stevens DL, Kaplan EL, eds. *Streptococcal Infections: Clinical Aspects, Microbiology, and Molecular Pathogenesis*. Oxford, UK: Oxford University Press, 2000; 222–37.

2 Farley MM, Harvey RC, Stull T *et al.* A population-based assessment of invasive disease due to group B *Streptococcus* in nonpregnant adults. *N Engl J Med* 1993; **328**: 1807–11.

3 Heath PT, Balfour G, Weisner AM *et al.* Group B streptococcal disease in UK and Irish infants younger than 90 days. *Lancet* 2004; **363**: 292–4.

4 Jackson LA, Hilsdon R, Farley MM *et al.* Risk factors for group B streptococcal disease in adults. *Ann Intern Med* 1995; **123**: 415–20.

5 Schuchat A. Group B *Streptococcus*. *Lancet* 1999; **353**: 51–6.

6 CDC. Prevention of perinatal group B streptococcal disease. *MMWR Recomm Rep* 2002; **51**: 1–22.

7 Schuchat A. Epidemiology of group B streptococcal disease in the United States: shifting paradigms. *Clin Microbiol Rev* 1998; **11**: 497–513.

8 Stoll BJ, Hansen N, Fanaroff AA *et al.* Changes in pathogens causing early-onset sepsis in very-low-birth-weight infants. *N Engl J Med* 2002; **347**: 240–7.

9 Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Sixteenth Informational Supplement M100-S16*. CLSI, Wayne, PA, USA, 2006.

10 Kimura K, Suzuki S, Wachino J *et al.* First molecular characterization of group B streptococci with reduced penicillin susceptibility. *Antimicrob Agents Chemother* 2008; **52**: 2890–7.

11 Nagano N, Nagano Y, Kimura K *et al.* Genetic heterogeneity in *pbp* genes among clinically isolated group B streptococci with reduced penicillin susceptibility. *Antimicrob Agents Chemother* 2008; **52**: 4258–67.

12 Nagano N, Kimura K, Nagano Y *et al.* Molecular characterization of group B streptococci with reduced penicillin susceptibility recurrently isolated from a sacral decubitus ulcer. *J Antimicrob Chemother* 2009; **64**: 1326–8.

13 Dahesh S, Hensler ME, Van Sorge NM *et al.* Point mutation in the group B streptococcal *pbp2x* gene conferring decreased susceptibility to β-lactam antibiotics. *Antimicrob Agents Chemother* 2008; **52**: 2915–8.

14 Gaudreau C, Lecours R, Ismaïl J *et al.* Prosthetic hip joint infection with a *Streptococcus agalactiae* isolate not susceptible to penicillin G and ceftriaxone. *J Antimicrob Chemother* 2010; **65**: 594–5.

15 Murayama SY, Seki C, Sakata H *et al.* Capsular type and antibiotic resistance in *Streptococcus agalactiae* isolates from patients, ranging from newborns to the elderly, with invasive infections. *Antimicrob Agents Chemother* 2009; **53**: 2650–3.

16 Jones N, Bohnsack JF, Takahashi S *et al.* Multilocus sequence typing system for group B *Streptococcus*. *J Clin Microbiol* 2003; **41**: 2530–6.

17 Kimura K, Wachino J, Kurokawa H *et al.* Practical disk diffusion test for detecting group B *Streptococcus* with reduced penicillin susceptibility. *J Clin Microbiol* 2009; **47**: 4154–7.

18 Lin FC, Whiting A, Adderson E *et al.* Phylogenetic lineages of invasive and colonizing strains of serotype III group B streptococci from neonates: a multicenter prospective study. *J Clin Microbiol* 2006; **44**: 1257–61.

19 Luan S, Granlund M, Sellin M *et al.* Multilocus sequence typing of Swedish invasive group B *Streptococcus* isolates indicates a neonatally associated genetic lineage and capsule switching. *J Clin Microbiol* 2005; **43**: 3727–33.

20 Manning SD, Cody Springman A, Lehotzky E *et al.* Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. *J Clin Microbiol* 2009; **47**: 1143–8.

21 Martins ER, Pessanha MA, Ramirez M *et al.* Analysis of group B streptococcal isolates from infants and pregnant women in Portugal revealing two lineages with enhanced invasiveness. *J Clin Microbiol* 2007; **45**: 3224–9.